ISOTOPE DERIVATIVE METHOD
FOR
QUANTITATIVE DETERMINATION
OF
HISTAMINE

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QUANTITATIVE DETERMINATION OF HISTAMINE

Abstract of

A Thesis

Presented in Partial Fulfillment of the Requirements

for the Degree Master of Science

by

Richard Raphael Entwhistle, Ch. E.

The Ohio State University

1952

Approved by:

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Adviser

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ISOTOPE DERIVATIVE METHOD
FOR
QUANTITATIVE DETERMINATION OF HISTAMINE

RICHARD RAPHAEL ENTWHISTLE

CH.E., UNIVERSITY OF CINCINNATI, 1935

Department of Physics
(Approved by William G. Myers)

A review of various methods for separating histamine from interfering substances and the quantitative determination of histamine by chemical methods is presented. The isotope derivative method of analysis is then described and its adoption as a method for the quantitative determination of histamine is discussed. Detailed methods, together with flow diagrams, are presented for the preparation of pipsyl-chloride, radioactive pipsyl-chloride, and the pipsyl-histamine derivative. By the method described herein, pipsyl-chloride tagged with S-thirty-five was prepared at the five thousandths mole level from radioactive sulfanilic acid. The pipsyl-chloride contained forty-four percent of the activity of the sulfanilic acid. Two equivalents of pipsyl-chloride appeared to combine with one equivalent of histamine to form a derivative which was difficult to dissolve. Of the many solvents tested, only chloroform, toluene and acetone dissolved the pipsyl-histamine derivative. The solubility was approximately one part of the pipsyl-histamine derivative to one-thousand parts of solvent.

The isotope derivative method using the carrier technic and chloroform as the solvent was applied to a sample containing seventy-five hundredths of a microgram of histamine. The histamine values as determined by this method were high by a factor of two-hundred to four-hundred percent, indicating the presence of radioactive contaminants. Activity measurements showed that self-absorption of the weak beta-particle emitted by S-thirty-five renders the use of the radioactive sulfur isotope undesirable as a tag in this method of analysis unless a correction factor, a function of the surface density, is applied. Histamine was separated from histidine and arginine by paper chromatography using isopentanol saturated with two normal ammonium hydroxide as a developer; the R_f factors for histamine, arginine and histidine were forty-one hundredths, zero and zero, respectively.

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I. INTRODUCTION:

Histamine and histamine-like substances have been named as the probable causative agents in the response of the body to anaphylactic shock, serum sickness, allergy, injury to cold (1), exposure to ionizing radiation (2), and other intrinsic effects. Perhaps much of the doubt as to whether histamine is the causative agent results from the fact that there is no quick simple accurate method for detecting histamine in low concentrations. Numerous estimates have been made of the amounts of histamine in animal tissue. Blood, however, has received the most thorough study because of the ease of obtaining samples and the fact that successive samples can be taken in the course of an observation. Since the histamine content of blood is rather low and deviations from the normal are small, an extremely sensitive method of detection must be employed. In order better to understand the order of sensitivity demanded, the concentration of histamine in micrograms per ml. of rabbit blood, which has the highest concentration, and of a normal man are cited as about 1.25 and 0.05, respectively (3). While the main research effort relative to histamine determination has been devoted to blood with the major emphasis on the separation of histamine from interfering substances and improving the sensitivity at the sub-microgram level, the technics developed are not restricted to blood analysis alone.

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A search of the literature reveals that purely chemical methods for the detection and quantitative determination of histamine have been

devised. Each of these methods employed a preliminary purification or separation of the histamine followed by a spectrophotometric determination. The following methods of preliminary purification have been employed:

a. Precipitation and extraction procedures (4, 5, 6).

b. Adsorption on synthetic resins, cotton acid succinate (6, 7, 8), amberlite (9), and by paper chromatography (10).

In the final determination of histamine, color reactions involving diazotization (Pauly reaction) with p-Diazobenzene sulfonate (4), p-Bromoaniline (5, 10), or 4-Nitroaniline (6, 9) have been employed, or optical densities have been measured after the reaction of histamine with 2, 4-Dinitrofluorobenzene (11). The earlier procedures lacked sufficient sensitivity for the detection of histamine concentrations normally found in small volumes of blood.

The most widely used and accepted procedures involve some initial purification procedure of the histamine followed by its biological assay. The bioassay method has, in general, been more sensitive than colorimetry in the quantitative estimation of histamine but it has also been found to be less accurate (12). The initial chemical procedures have been directed toward liberating histamine from substances and structures with which it is bound, separating it from other similar substances which would interfere with its accurate biological determination or accurate chemical determination while simultaneously maintaining a sufficiently

constant and high yield of the histamine originally present to allow a satisfactory quantitative estimation. The final biological assay employing guinea pig gut, uterus or bronchi must be sufficiently sensitive to detect the minute quantities of histamine normally present (13).

II. ISOTOPIC DERIVATIVE METHOD:

1. General: It is apparent that the presently accepted and employed methods all depend on an initial chemical separation of histamine from interfering substances before the final quantitative determination is made. This procedure has its analogue in gravimetric analysis whereby a desired compound is quantitatively precipitated from solution. In employing the latter procedure it is realized that a small amount of the compound sought, limited by the solubility product, is lost to the solution. When analyzing for macro-amounts of material, the amount lost to the solution usually contributes a negligible error in the final result. However, when dealing with micro-quantities, i.e., at the microgram and sub-microgram level, the amount of material not recovered by the extraction may be large enough to render worthless the final result of the analysis. Since the isotope derivative method, using the carrier technic, would eliminate this source of error, it was chosen for exploration as a possible method for the quantitative detection of histamine. The isotope derivative method lends itself admirably to the present situation since it has been demonstrated to have an extremely high sensitivity, being operable below the microgram level (14).

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2. Outline of Method: In brief, this method consists of the following steps (14):

a. Treating the unknown mixture with a reagent containing a radioactive isotope under such conditions that the component to be estimated is quantitatively converted to the derivative of radioactive reagent.

b. Adding a large excess, accurately weighed, of the unlabelled derivative to the unknown mixture.

c. Separating and purifying the desired derivative to a constant molal isotope concentration. (Note: This doesn't imply that the derivative must be recovered quantitatively. However, that fraction which is recovered must be isotopically pure, i.e., counts per mole per second for successive fractions must be constant).

d. Preparing an isotopic derivative, using the same isotopically labelled reagent employed in step "a", of a known amount of the component sought and purifying to constant molal isotopic concentration.

e. Determining the quantity of the desired component present in the unknown mixture by comparison of the constant specific molal activities according to the following formula:

$$w = \frac{U(w + W)}{K}$$

where,

w = amount of isotopic derivative which was present in the unknown mixture.

W = amount of unlabelled derivative (carrier) added in excess.

U = constant molal isotope concentration resulting from unknown mixture.

K = constant molal isotope concentration resulting from reaction involving the known amount of the component sought.

When relatively large amounts of carrier are added, the formula reduces to the form:

$$w = \frac{WU}{K}$$

Brief and simple though the procedure may be, it is emphasized that in order for the final result to be meaningful (14):

a. The reaction between the isotopic reagent and the compound sought must be complete.

b. The carrier which is isolated from the reaction mixture must be rigorously purified from radioactive contaminants.

c. The measurement of the quantities U and K must be precise.

III. CHOICE OF ISOTOPICALLY TAGGED REAGENT:

Acid chlorides were decided upon as the reagents which would be used to react with histamine. It was assumed a priori that histamine would react with acid chlorides in the same manner as the amino acids (14) since it contained a primary and secondary reactive amino-group.

2, 6-diiodosulfanilic acid, provided it could be converted to 2,6-diiodobenzene sulfonyl chloride, was suggested since it could be prepared at the 0.001 mole level in high yield (85%), an important factor in preparing radioactive compounds. The diiodobenzene sulfonyl chloride

Y = constant molal isotope concentration resulting from unknown mixture.

Z = constant molal isotope concentration resulting from reaction involving the known amount of the component sought.

When relatively large amounts of carrier are added, the formula reduces to the form:

$$x = \frac{YD}{Z}$$

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tagged with I-131 would prove an excellent reagent because it could be prepared with an extremely high specific activity due to the two (2) iodine atoms, representing 59% by weight, which are an integral part of the molecule. The high specific activity would permit a greater sensitivity in detecting components with which it reacts. Efforts to replace the amino-group of the diiodosulfanilic acid by hydrogen,prior to conversion to an acid chloride, resulted in failure.

The methods employed to replace the amino-group with hydrogen was first to diazotize the diiodosulfanilic acid and then treat with excess hypophosphorous acid (15) or 95% ethanol (16). The resulting products showed evidence of iodine decomposition upon recrystallization from aqueous or alcohol solution at temperatures as low as 50° C. The recovered product had a melting point range 121 - 180° C, indicating a mixture of components.

It was finally decided to use pipsyl-chloride (p-iodobenzene sulfonyl chloride) as the reagent to react with histamine since the literature indicated that this compound could be prepared and isolated in pure form.

IV. PREPARATION OF PIPSYL-CHLORIDE (P-IODOBENZENE SULFONYL CHLORIDE):

1. Non-radioactive: Reactions of the type required to prepare compounds similar to pipsyl-chloride were found in the literature (17,18). However, since there were no specific procedures for the preparation of pipsyl-chloride, the steps are outlined herein: (Reference is flow sheet No. 1 for diagram of process.)

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a. Dissolve sulfanilic acid in 8% NaOH, add $NaNO_2$ and run slowly with stirring into a mixture of 20% H_2SO_4 and ice.

$$C_6H_4(NH_2)SO_3H + NaOH \longrightarrow C_6H_4(NH_2)SO_2ONa + H_2O$$

$$2C_6H_4(NH_2)SO_2ONa + 2\,NaNO_2 + 3H_2SO_4 \rightarrow 2Na_2SO_4 + 4H_2O + (C_6H_4(N_2)SO_3H)_2SO_4$$

b. Permit diazotized compound to stand 1 hour in order to settle out.

c. Decant off supernatant liquid in order to eliminate excess $NaNO_2$ used in diazotization. (Note 1)

d. Add a concentrated solution of KI to diazotized salt and permit Sandmeyer reaction to take place at room temperature. Complete reaction by placing in a boiling water bath.

$$(C_6H_4(N)_2SO_3H)_2SO_4 + 2KI \longrightarrow 2C_6H_4(I)SO_3H + 2N_2 + K_2SO_4$$

e. Permit reaction mixture to cool, make alkaline with NaOH, and salt out of sodium salt of p-iodobenzene sulfonate with NaCl.

$$C_6H_4(I)SO_3H + NaOH \longrightarrow C_6H_4(I)SO_2ONa + H_2O$$

f. Filter through Buchner funnel and dry at 140° C for 3 hours.

g. Mix finely powdered sodium p-iodobenzene sulfonate with PCl_5 and $POCl_3$ in ratio of 1 mole of salt to 0.8 moles of $POCl_3$ and 0.3 moles of PCl_5. Reflux mixture at 170 - 180° C for 16 hours. Cool reaction mixture for 5 minutes at the end of each 4 hour period and shake until mixture becomes pasty. The shaking brings the unreacted components together and thereby increases the yield. (Note 2)

a. Dissolve sulfanilic acid in 5% NaOH, add $NaNO_2$ and run slowly with stirring into a mixture of 30% H_2SO_4 and ice.

$$C_6H_4(NH_2)SO_3H + NaOH \longrightarrow C_6H_4(NH_2)SO_2ONa + H_2O$$

$$2C_6H_4(NH_2)SO_2ONa + 2\,NaNO_2 + 3H_2SO_4 \longrightarrow 2Na_2SO_4 + 4H_2O$$
$$+(C_6H_4(N_2)SO_3H)_2SO_4$$

b. Permit diazotized compound to stand 1 hour in order to settle out.

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$$C_6H_4(I)SO_3H + NaOH \longrightarrow C_6H_4(I)SO_2ONa + H_2O$$

f. Filter through Buchner funnel and dry at 140° C for 3 hours.

g. Mix finely powdered sodium p-iodobenzene sulfonate with PCl_5 and $POCl_3$ in ratio of 1 mole of salt to 0.9 moles of $POCl_3$ and 4.2 moles of PCl_5. Reflux mixture at 170 - 180° C for 18 hours. Cool reaction mixture for 5 minutes at the end of each 4 hour period and shake until mixture becomes pasty. The shaking brings the unreacted components together and thereby increases the yield. (Note 2)

$$3C_6H_4(I)SO_2ONa + PCl_5 \longrightarrow 3C_6H_4(I)SO_2Cl + 2NaCl + NaPO_3$$

$$2C_6H_4(I)SO_2ONa + POCl_3 \longrightarrow 2C_6H_4(I)SO_2Cl + NaCl + NaPO_3$$

h. Cool reaction mixture and extract with benzene, grinding the solid material with the benzene to facilitate the extraction.

i. Wash the benzene fraction 3 times with ice water to remove dissolved unreacted phosphorous halides and inorganic salts.

j. Dry benzene fraction over anhydrous Na_2SO_4.

k. Evaporate to dryness on a water bath and dissolve in a slight excess of ethyl ether.

l. Add activated carbon and warm for a few minutes to remove color.

m. Filter through Buchner funnel to remove carbon.

n. Reduce volume of ether to about one-half by evaporation and place in ice bath. Crystals of pipsyl-chloride now appear.

o. Filter off pipsyl-chloride and wash crystals with petroleum ether. Pipsyl-chloride is insoluble in petroleum ether.

p. Add petroleum ether wash to mother liquor and again reduce volume by about one-half. Cool and pipsyl-chloride crystals separate out. Wash crystals with petroleum ether. This procedure was repeated until a pink oily liquid instead of pipsyl-chloride crystals separated out on cooling. The pink oily liquid was discarded.

Note 1: This reduces the final yield of p-iodobenzene sulfonic acid

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but it eliminated the necessity of adding urea to remove excess $NaNO_2$ before addition of KI.

Note 2: This ratio of phosphorous halides to p-iodobenzense sulfonic acid gives the best yield of the sulfonyl chloride.

Each batch of crystals was kept separate and their melting point determined. The melting point for each batch ranged between 84°-85° C (literature 86-87°C) (19). To identify the crystals further as pipsyl-chloride, they were reacted with glycine and alanine. Melting points of the glycine derivative and alanine derivative were found to be 204 -205° C and 194 -195°C, respectively. The literature reports the melting point of glycine and alanine derivatives of pipsyl-chloride to be 205°C and 194.5°C, respectively (14). Thus it can be concluded that the product was pipsyl-chloride. The yield of pipsyl-chloride based upon the original amount of sulfanilic acid used was 59%.

2. Radioactive:

a. Preparation: Sulfanilic acid tagged with S-35 was used as the starting product because this reagent was made available in the laboratory. The procedure for converting sulfanilic acid to p-iodobenzene sulfonyl-chloride at the 0.0005 mole level is given in detail below. A "cold" run was conducted in parallel with the "hot" run to act as a control at each stage of the process. The sulfanilic acid (0.09 grams) tagged with 7.75 millicuries of S-35 was received as the sodium salt dissolved in 25 ml. of water.*(Reference is flow sheet No. 2 for diagram of process.)

* Radioactive sulfanilic acid was prepared by David Imhof, a student in the Arts and Science College, Ohio State University.

but it eliminated the necessity of adding urea to remove excess HNO_2 before addition of KI.

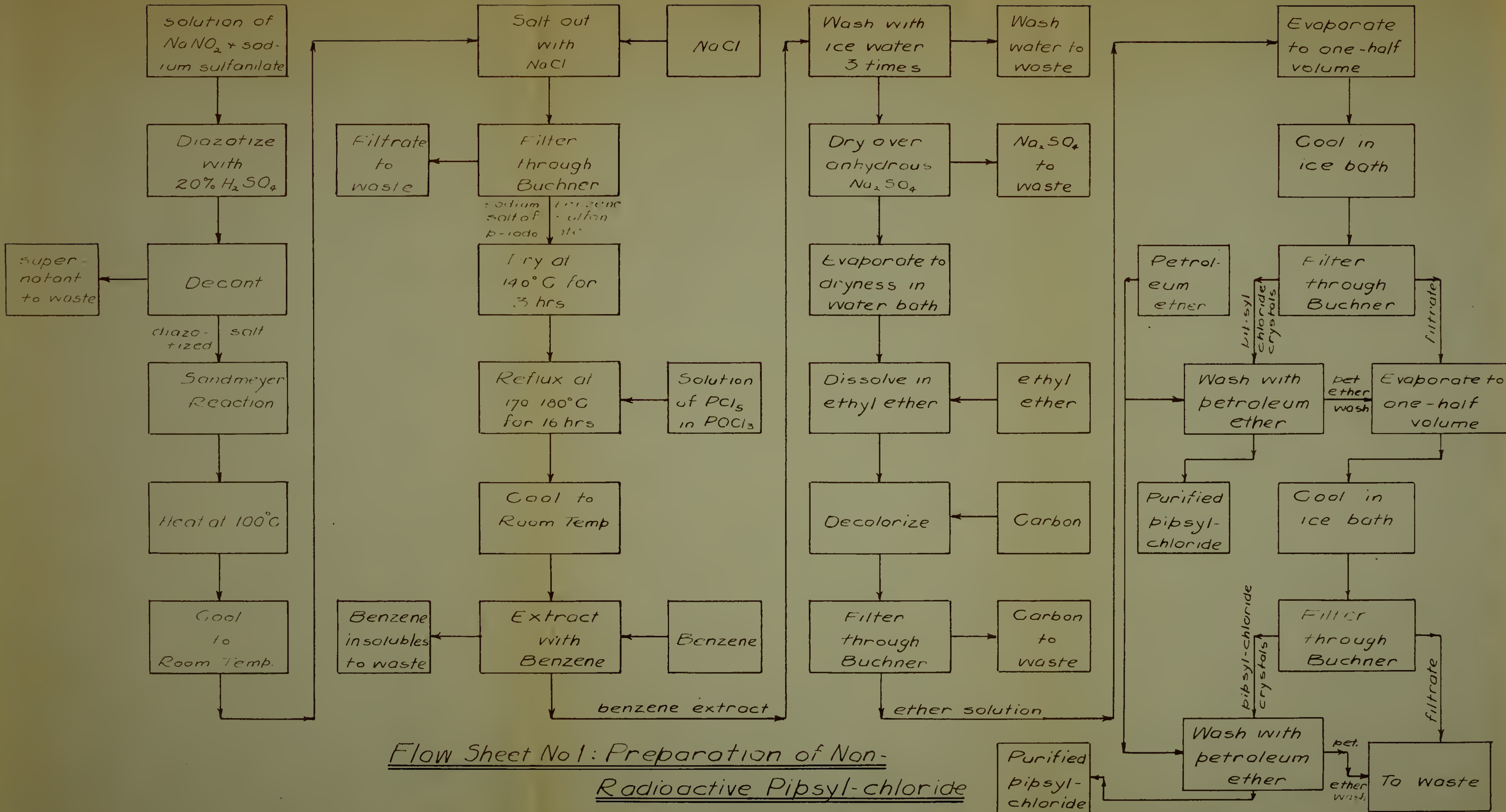
Note 2: This ratio of phosphorous iodide to p-acetamidobenzene sulfonic acid gave the best yield of the sulfonyl chloride.

Each batch of crystals was kept separate and their melting point determined. The melting point for each batch ranged between 94°-95° C (literature 95-97°C) (14). To identify the crystals further as pipsyl-chloride, they were reacted with glycine and alanine. Melting points of the glycine derivative and alanine derivative were found to be 204-205°C and 194-195°C, respectively. The literature reports the melting point of glycine and alanine derivatives of pipsyl-chloride to be 201°C and 194.5°C, respectively (14). Thus it can be concluded that the product was pipsyl-chloride. The yield of pipsyl-chloride based upon the original amount of sulfanilic acid used was 59%.

2. Radioactive:

a. Preparation: Sulfanilic acid tagged with S-35 was used as the starting product because this reagent was made available in the laboratory.* The procedure for converting sulfanilic acid to p-iodobenzene sulfonyl-chloride at the 0.1 mole scale level is given in detail below. A "cold" run was conducted in parallel with the "hot" run to act as a control at each stage of the process. The sulfanilic acid (0.05 grams) tagged with 7.75 millicuries of S-35 was received as the sodium salt dissolved in 75 ml. of water. *(Reference is flow sheet No. 2 for diagram of process.)

* Radioactive sulfanilic acid was prepared by David Imbot, a student in the Arts and Science College, Ohio State University.



Flow Sheet No 1: Preparation of Non-Radioactive Pipsyl-chloride

(1) Place sodium sulfanilate solution in 40 ml. centrifuge tube.

(2) Evaporate volume to 3 ml., frequently washing down the sides of the tube with hot distilled water.

(3) Add 0.0375 g. $NaNO_2$ (very slight excess over stoichiometric amount) dissolved in 0.25 ml. distilled water.

(4) Add the mixture of the sodium sulfanilate and $NaNO_2$ dropwise to a 40 ml. centrifuge tube containing 0.5 ml. 20% H_2SO_4 maintained at 0-5° C. The sulfuric acid was stirred vigorously during the addition of the mixture and stirring was continued for 30 minutes to insure completeness of reaction. The tube containing the sodium sulfanilate was rinsed carefully, using a total of 3 ml. of distilled water.

(5) Permit diazotized salt to stand for 1 hour at 10° C.

(6) Add 0.0935 g. KI (excess over stoichimetric requirement) and permit to react 1 hour at room temperature. Complete reaction by placing in boiling water bath for 15 minutes.

(7) Evaporate reaction mixture to 2 ml. and add C.P. NaCl to salt out p-iodobenzene sulfonic acid.

(8) Centrifuge mixture for 5 minutes to throw down p-iodobenzene sulfonic acid, pipet off mother liquor and wash

(1) Place sodium sulfanilate solution in 40 ml. centrifuge tube.

(2) Evaporate volume to 1 ml., frequently washing down the sides of the tube with hot distilled water.

(3) Add 0.0572 g. $NaNO_2$ (very slight excess over stoichiometric amount) dissolved in 0.25 ml. distilled water.

(4) Add the mixture of the sodium sulfanilate and $NaNO_2$ dropwise to a 40 ml. centrifuge tube containing 0.2 ml. 30% H_2SO_4 maintained at 0-5° C. The sulfuric acid was stirred vigorously during the addition of the mixture and stirring was continued for 30 minutes to insure completeness of reaction. The tube containing the sodium sulfanilate was rinsed carefully, using a total of 2 ml. of distilled water.

(5) Permit diazotized salt to stand for 1 hour at 10° C.

(6) Add 0.0953 g. KI (excess over stoichiometric requirement) and permit to react 1 hour at room temperature. Complete reaction by placing in boiling water bath for 15 minutes.

(7) Evaporate reaction mixture to 2 ml. and add C.P. NaCl to salt out p-iodobenzene sulfonic acid.

(8) Centrifuge mixture for 5 minutes to throw down p-iodobenzene sulfonic acid, pipet off mother liquor and wash

precipitate with 1 ml. cold saturated brine.

(9) Centrifuge tube containing precipitate for 5 minutes and pipet off supernatant liquid. Add supernatant to mother liquor and set aside p-Iodobenzene for drying.

(10) Warm mixture of supernatant from previous step and mother liquor, and add 0.04 g. p-Iodobenzene sulfonic acid (non-radioactive) as a saturated solution in warm water. Mix and cool to 5° C. p-Iodobenzene sulfonic acid precipitates out.

(11) Centrifuge mixture for 5 minutes; pipet off supernatant; wash precipitate with 1 ml. cold saturated brine; again centrifuge; pipet off supernatant and combine it with mother liquor; and set aside p-Iodobenzene sulfonic acid for drying.

(12) Repeat steps 10 and 11 once more. The final wash water was still slightly radioactive.

(13) Dry the 3 precipitates at 120° C. for 3 hours.

(14) Add 1 ml. $POCl_3$ saturated with PCl_5 to each of the 3 tubes and pool contents of the 3 tubes into the one containing the first precipitated p-Iodobenzene sulfonic acid. Carefully wash the tubes with a total of 1 ml. of the phosphorous halide mixture to insure complete transfer.

(15) Heat mixture in an oil bath at 100 - 110° C. for 5 hours.

(16) Cool reaction mixture and transfer it to a separatory funnel containing 50 ml. benezene and 25 ml. ice water. Use a small volume of benzene to make the transfer.

(17) Add 0.2 g. pure non-isotopic pipsyl-chloride to the mixture of benzene and water.

(18) Wash the benzene layer 3 times, using 15 - 20 ml. ice water for each wash. The final wash water was only slightly radioactive.

(19) Add approximately 20 g. anhydrous Na_2SO_4 to benzene fraction and allow to stand 12 hours to remove water.

(20) Filter off benzene fraction and wash Na_2SO_4 10 times, using a total of 100 ml. benzene. Na_2SO_4 still remained quite radioactive.

(21) Evaporate benzene fraction to one-half original volume.

(22) Again wash Na_2SO_4 5 times, using a total of 50 ml. hot benzene, and add to benzene fraction in step 21. Na_2SO_4 showed a marked decrease in radioactivity.

(23) Return benzene fraction to water bath at 60 - 65° C. and evaporate to dryness.

(24) Wash down sides of beaker with 10 ml. ethyl ether; add approximately 0.3 g. activated carbon; filter; wash carbon with 5 ml. ethyl ether; and evaporate to dryness. Colorless crystals now appear.

(15) Heat mixture in an oil bath at 100 - 110° C. for 6 hours.

(16) Cool reaction mixture and transfer it to a separatory funnel containing 50 ml. benzene and 20 ml. ice water. Use a small volume of benzene to make the transfer.

(17) Add 0.5 g. pure non-isotopic phenyl-chloride to the mixture of benzene and water.

(18) Wash the benzene layer 3 times, using 15 - 20 ml. ice water for each wash. The final wash water was only slightly radioactive.

(19) Add approximately 20 g. anhydrous Na_2SO_4 to benzene fraction and allow to stand 12 hours to remove water.

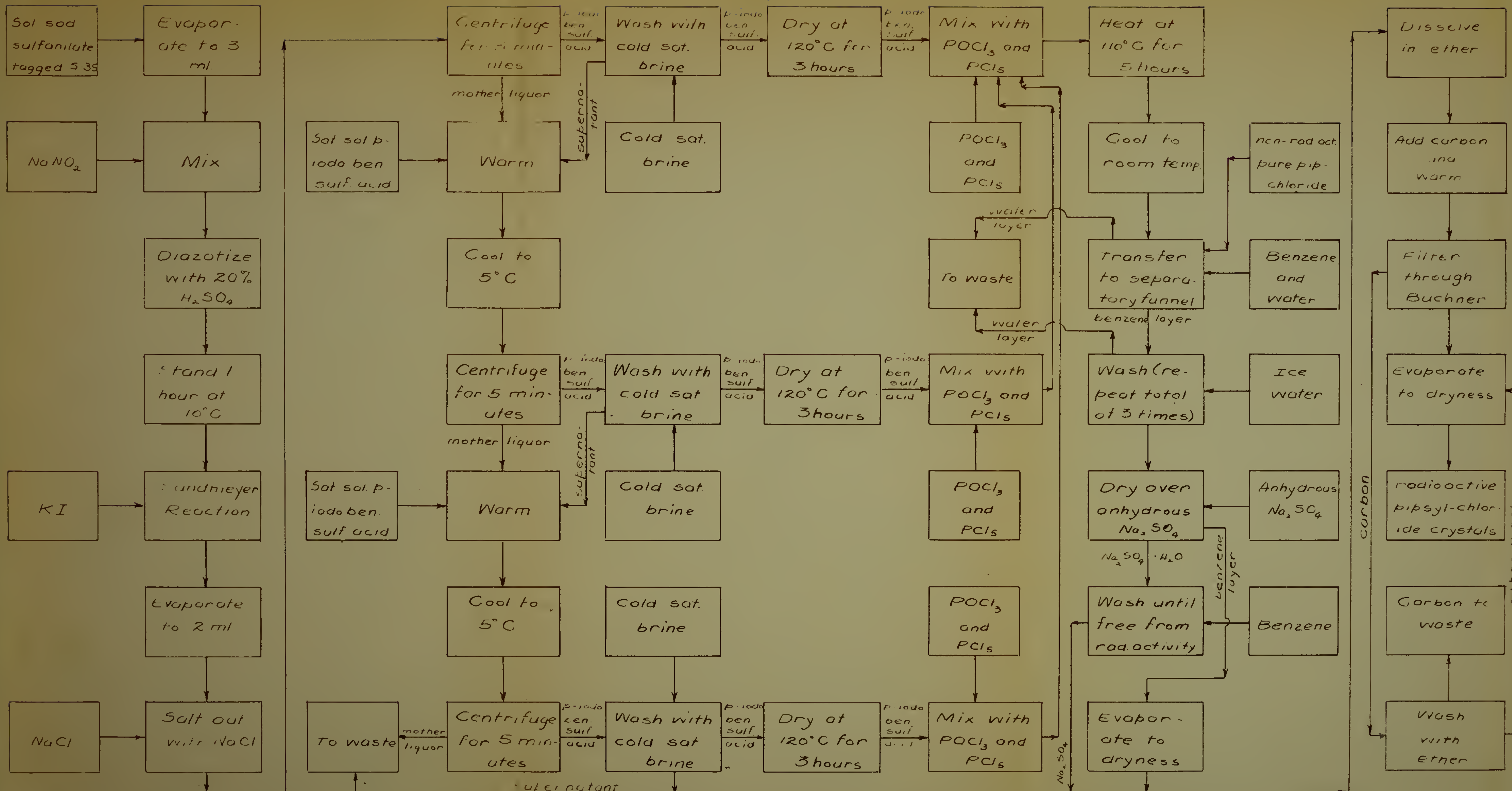
(20) Filter off benzene fraction and wash Na_2SO_4 10 times, using a total of 150 ml. benzene. Na_2SO_4 still remained quite radioactive.

(21) Evaporate benzene fraction to one-half original volume.

(22) Again wash Na_2SO_4 5 times, using a total of 50 ml. hot benzene, and add to benzene fraction in step 21. Na_2SO_4 showed a marked decrease in radioactivity.

(23) Return benzene fraction to water bath at 50 - 55° C. and evaporate to dryness.

(24) Wash down sides of beaker with 10 ml. ethyl ether; add approximately 0.2 g. activated carbon; filter; wash carbon with 5 ml. ethyl ether; and evaporate to dryness. Colorless crystals now appear.



Flow Sheet No.2 : Preparation of Radioactive Pipsyl-chloride

The carbon residue from the final step of the recovery of the pipsyl-chloride remained extremely radioactive despite repeated washings with benzene and ether (these washings were not added to the final product). This high activity is understandable from the fact that 0.087 g. of product was retained by the carbon. The final yield of pipsyl-chloride was 0.301 g.

b. Assay: The radioactive pipsyl-chloride was assayed by weighing out a small sample, dissolving it in benzene, taking aliquots, evaporating to dryness on an aluminum planchet, and counting by Model SC 1 Autoscaler, Tracerlab, using flow chamber with gas mixture 99% helium and 0.95% isobutane.

Mg. Sample Used	Net Count	Seconds	Counts per mg. per sec. $\times 10^4$
0.019	4067	17.4	1.24
0.0095	4042	31.6	1.34
0.0019	3871	133.5	1.53
			Ave 1.37

The amount of radioactivity incorporated into the recovered pipsyl-chloride, assuming a counting efficiency of 33% due to geometry, is:

Amount of radioactivity incorporated into pipsyl-chloride $= \dfrac{1.37\times10^4 \times 3.01\times10^2}{3.3\times10^{-1}}$

$= 12.5 \times 10^6$ counts per sec.

number of millicuries represented by sample $= \dfrac{12.5 \times 10^6}{3.7 \times 10^6} = 3.4$

The carbon residue from the final step of the recovery of the pipsyl-chloride remained extremely radioactive despite repeated washings with benzene and ether (these washings were not added to the final product). This high activity is understandable from the fact that 0.097 g. of product was retained by the carbon. The final yield of pipsyl-chloride was 0.301 g.

c. Assay: The radioactive pipsyl-chloride was assayed by weighing out a small sample, dissolving it in benzene, taking aliquots, evaporating to dryness on an aluminum planchet, and counting by Model SC 1 Autoscaler, Tracerlab, using flow chamber with gas mixture 99% helium and 0.95% isobutane.

Mg. Sample Used	Net Count	Seconds	Counts per mg. per sec. $\times 10^4$
0.015	4087	17.4	1.34
0.0095	4049	31.5	1.34
0.0019	3871	133.5	1.43
			Ave 1.37

The amount of radioactivity incorporated into the recovered pipsyl-chloride, assuming a counting efficiency of 22% due to geometry, is:

$$\text{Amount of radioactivity incorporated into pipsyl-chloride} = \frac{1.37\times10^4 \times 2.01\times10^2}{2.2\times10^{-1}}$$

$$= 12.5 \times 10^6 \text{ counts per sec}$$

$$\text{number of millicuries represented by sample} = \frac{12.5 \times 10^6}{3.7 \times 10^6} = 3.4$$

Yield in terms of initial radio-activity incorporated	=	$\frac{3.4 \times 10^2}{7.75}$
into final product	=	44%

V. PREPARATION OF PIPSYL DERIVATIVES:

1. Preparation of Pipsyl-Histamine Derivative: The pipsyl-histamine derivative was prepared as follows:

a. Dissolve histamine dihydrochloride in water, and add enough $NaHCO_3$ to neutralize the HCl associated with the molecule and that which would be formed during the reaction.

b. Place solution in a boiling water bath and add, with stirring, excess pipsyl-chloride.

c. Stir vigorously for 30 minutes while maintaining the temperature near the boiling point.

d. To recover precipitate formed during step above,

(1) Decant off supernatant.

(2) Wash precipitate with ethyl ether to remove unreacted pipsyl-chloride and p-iodophenyl sulfonic acid arising from hydrolysis of excess pipsyl-chloride.

(3) Wash precipitate with water.

(4) Dry precipitate at 120° C for 2 hours.

Aliquots of the supernatant liquid recovered in step d (1) were acidified with dilute and concentrated HCl and H_2SO_4, and cooled to 0 °C. Only the slightest turbidity resulted. Extraction with ether, returning to a small volume of NH_4OH, acidification and cooling still resulted in

only a slight turbidity.

The reaction between histamine and pipsyl-chloride is wholly unlike that of the amino acids and pipsyl-chloride; the amino acid derivatives are soluble in a basic medium whereas the histamine derivative is insoluble in both acid and basic media. A precipitate was obtained regardless of whether histamine or pipsyl-chloride was used in excess. Applying the excess pipsyl-chloride in steps did not alter the reaction.

No attempt was made rigorously to identify the precipitate. However, it is believed to be a ternary salt resulting from the coupling of two equivalents of pipsyl-chloride with one equivalent of histamine. It was initially discovered that aromatic sulfonic acids act as precipitants for basic amino acids; later it was shown that sulfonic acids as a class will precipitate all amino acids, with the less basic amino acids usually requiring a more complex sulfonic acid (20). It is realized that histamine is not an amino acid but it is relatively basic and is structurally similar to histidine, a basic amino acid. Attempts to prepare a precipitate of histamine resulted in failure using benzene sulfonic acid and 2, 6-diiodobenzene sulfonic acid according to the established method of preparing pipsyl-chloride derivatives and that described by Doherty, et al (21). Another reason for believing that two equivalents of histamine react with one equivalent of pipsyl-chloride are the stoichiometric relationships involved which are summarized below.

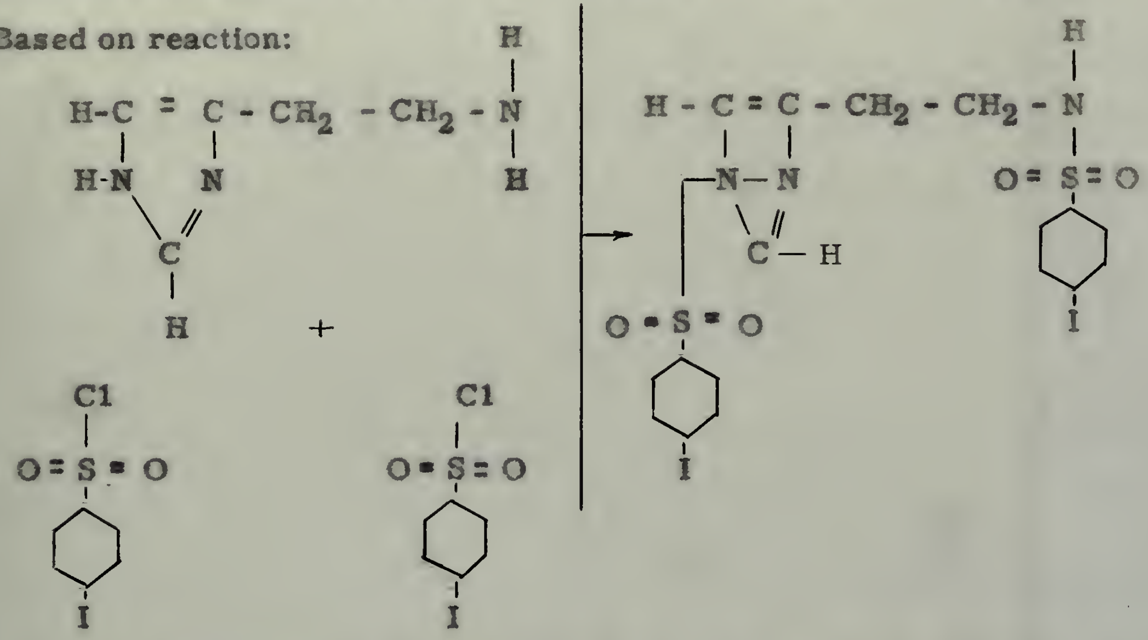
only a slight turbidity.

The reaction between histamine and pipsyl-chloride is wholly unlike that of the amino acids and pipsyl-chloride; the amino acid derivatives are soluble in a basic medium whereas the histamine derivative is insoluble in both acid and basic media. A precipitate was obtained regardless of whether histamine or pipsyl-chloride was used in excess. Applying the excess pipsyl-chloride in steps did not alter the reaction.

No attempt was made rigorously to identify the precipitate. However, it is believed to be a tertiary salt resulting from the coupling of two equivalents of pipsyl-chloride with one equivalent of histamine. It was initially discovered that aromatic sulfonic acids act as precipitants for basic amino acids; later it was shown that sulfonic acids as a class will precipitate all amino acids, with the less basic amino acids usually requiring a more complex sulfonic acid (20). It is realized that histamine is not an amino acid but it is relatively basic and is structurally similar to histidine, a basic amino acid. Attempts to prepare a precipitate of histamine resulted in failure using benzene sulfonic acid and 2, 6-diiodobenzene sulfonic acid according to the established method of preparing pipsyl-chloride derivatives and that described by Doherty, et al (21). Another reason for believing that two equivalents of histamine react with one equivalent of pipsyl-chloride are the stoichiometric relationships involved which are summarized below.

	Amount in Excess	Actual wt. * ppt. recovered / theoretical wt. ppt. resulting from reaction	Melting Point °C	Test for Histamine in Supernatant**
Excess histamine	50%	0.88	169-170	Pos.
Excess pipsyl-chloride	45%	0.875	169	Neg.

*Based on reaction:



** A drop of supernatant was placed on No. 2 Whatman filter paper, dried for 1 hour at 110°C, sprayed with a solution of ninhydrin (0.1% dissolved in n-butanol), and dried at 80°C for 5 minutes. A purple color resulted.

2. Determination of Solvent for Derivative:

a. General: It was necessary to find a suitable solvent for the pipsyl-histamine derivative since the extract of the analysis mixture must be added to the carrier before proceeding with the analysis. The search for a solvent was a difficult task for it figures critically in the separation of the desired derivative from contaminants. The criteria to be met by the solvent are:

(1) Dissolve pipsyl-histamine derivative readily.

(2) Immiscible with water or ethyl ether since the pipsyl amino acid derivatives, which would usually be present in an analysis, are soluble in these media.

(3) Insoluble to p-iodophenyl sulfonic acid resulting from hydrolysis of excess pipsyl-chloride used in the reaction.

(4) Not react with pipsyl-chloride to form a contaminating product. (This eliminates the alcohols).

(5) Permit ready recovery of the pipsyl-histamine carrier after separation has been performed.

While criteria (2) through (5) are not absolutely essential, they greatly simplify and facilitate the recovery of the pipsyl-histamine derivative in pure form.

b. Solvents: Precise quantitative measurements of the solubility of the pipsyl-histamine derivative in the various solvents were not made; only qualitative answers were sought. The solubilities, stated qualitatively, are as follows:

(1) Insoluble in hot and cold: water; dilute and concentrated NH_4OH, NaOH, HCl, H_2SO_4; benzene; ethyl ether; petroleum ether; carbon tetrachloride; butyl acetate; n-butyl ether; and ethyl butyl acetate.

(2) Slightly soluble in cold: acetone, chloroform and toluene.

(3) Moderately soluble in hot: acetone, chloroform and toluene.

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(2) Immiscible with water or ethyl ether since the pipsyl amino acid derivatives, which would usually be present in an analysis, are soluble in these media.

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(4) Not react with pipsyl-chloride to form a contaminating product. (This eliminates the alcohols).

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(1) Insoluble in hot and cold: water; dilute and concentrated NH_4OH, NaOH, HCl, H_2SO_4; benzene; ethyl ether; petroleum ether; carbon tetrachloride; butyl acetate; n-butyl ether; and ethyl butyl acetate.

(2) Slightly soluble in cold: acetone, chloroform and toluene.

(3) Moderately soluble in hot: acetone, chloroform and toluene.

No really good solvent, of those tested, was found. Acetone was the best of the three moderately good solvents, however, the fact that it is miscible with water makes it undesirable. Chloroform and toluene were equally good solvents, dissolving 1 g. of pipsyl-histamine derivative per 1000 g. solvent at 20° C. Both chloroform and toluene meet the criteria established in all but one respect, i.e., they both dissolve p-iodophenyl sulfonic acid to an appreciable extent. This may not necessarily make them unsuitable as solvents. However, when radioactive pipsyl-chloride was hydrolyzed in $NaHCO_3$ solution, acidified, extracted with chloroform, and counted; the aqueous fraction was slightly less radioactive than the chloroform fraction, indicating a slight preference of the sulfonic acid for the aqueous layer.

3. Preparation of Pipsyl-Derivatives of Di-amino Acids: Since the available quantity of histamine was rather limited and the amount of histidine relatively abundant, it was considered expedient to gain experience preparing the pipsyl-chloride derivative using histidine rather than histamine (assuming that because of their structural similarity the two compounds would react alike to pipsyl-chloride). The results of this diversion from the main problem proved to be interesting. Pipsyl derivatives of histidine were prepared according to the procedure used by Keston, Udenfriend and Cannan (14) using either an excess of amino acid or pipsyl-chloride. It was always possible to obtain a precipitate upon acidification of the reaction mixture of histidine and pipsyl-chloride but

the precipitate could not be recovered. The precipitate would disappear if attempts were made to recover it immediately, either by filtration or centrifugation at -5° C, or reduce itself to a small tan sticky mass if permitted to stand several hours at 0° C. Acidification with various acids; dilute and concentrated HCl, H_2SO_4, and $HC_2H_3O_2$; produced similar results. Precipitates would not appear unless the pH was reduced to a range 5.2 - 5.6; acidification beyond this range did not assist in the recovery. The pipsyl-chloride derivative of arginine behaved in a similar manner. It is to be noted that both histidine and arginine are di-amino acids. Accordingly, it appears as if pipsyl-chloride can be used successfully only with mono-amino acids.

VI. SEPARATION OF HISTAMINE FROM BASIC AMINO ACIDS BY PAPER CHROMATOGRAPHY:

1. Procedure: Histamine was separated from a solution of histamine, histidine and arginine on a paper chromatogram. The R_f factor for histamine was found to be 0.41; the R_f factor for both histidine and arginine, approximately zero. The experimental procedure used to effect the separation is described below:

a. Use a strip of Eaton-Dikeman filter paper, 1.5 cm. wide by 40-45 cm. long, for the chromatogram and a 500 ml. glass-stoppered graduate for the chamber.

b. Place a drop of solution containing about 5 micrograms of amino acid or histamine, 5 cm. from one end of the paper; designate this position as the zero-point. Weight the end of the paper on which

solution has been placed with a small lead weight.

c. Place paper, weighted end down, into chamber so that the lead weight just touches bottom. The bottom of the chamber contains 25 ml. of isopentanol saturated with 2N ammonium hydroxide. Secure other end of paper with glass stopper, being careful to keep paper vertical and away from side of graduate.

d. Develop for 10 hours at 23° C.

e. Remove paper from chamber and mark solvent front.

f. Dry paper in oven at 110° C.

g. Spray paper with ninhydrin (0.1% solution in n-butanol).

h. Air dry paper.

i. Heat paper at 80°C for 5 minutes to produce color.

j. Determine R_f factor by comparing the distance the center of each spot has moved from the zero-point to the distance the solvent front has moved from the zero-point.

When a mixture of components are to be separated, a drop of solution containing about 5 micrograms of each of the components is added to the paper strip and the procedure carried out as described above.

2. Determination of R_f Factors: The chromatographic separation produced the following results:

solution has been placed with a small lead weight.

c. Place paper, weighted end down, into chamber so that the lead weight just touches bottom. The bottom of the chamber contains 25 ml. of isopropanol saturated with 2N ammonium hydroxide. Secure other end of paper with glass stopper, being careful to keep paper vertical and away from side of graduate.

d. Develop for 12 hours at 22° C.

e. Remove paper from chamber and mark solvent front.

f. Dry paper in oven at 110° C.

g. Spray paper with ninhydrin (0.1% solution in n-butanol).

h. Air dry paper.

i. Heat paper at 80°C for 5 minutes to produce color.

j. Determine R_f factor by comparing the distance the center of each spot has moved from the zero-point to the distance the solvent front has moved from the zero-point.

When a mixture of components are to be separated, a drop of solution containing about 5 micrograms of each of the components is added to the paper strip and the procedure carried out as described above.

2. Determination of R_f Factors: The chromatographic separation produced the following results:

Added to Column	Distance of Solvent front from zero-point (cm.)	Distance of Center of colored spots from zero-point (cm.)	R_f Factor	Color of Spots Developed with Ninhydrin
Histamine	17.7	8.0	0.45	purple
Histamine	16.5	6.7	0.41	purple
Histidine	17.4	0	0	purple
Arginine	17.7	0	0	purple
Mixture of * histamine, histidine and arginine	17.9	0	0	purple
		7.5	0.41	purple

* Two purple spots appeared. The spot with $R_f = 0$ was due to histidine and arginine; the spot with $R_f = 0.41$ was due to histamine.

VII. EXPERIMENTAL PROCEDURE FOR ANALYSIS OF SAMPLE CONTAINING HISTAMINE: (Reference is Flow Sheet No. 3 for diagram of procedure.)

1. Pipet known amount of histamine solution (less than 0.6 ml.) where "U" is to be determined or unknown solution (less than 0.6 ml.) where "K" is to be determined into a Folin sugar tube.

2. Add 0.015 g. $NaHCO_3$.

3. Pipet 1 ml. of ether solution of radioactive pipsyl-chloride containing 0.010 g., tagged with about 0.02 millicurie S-35 into a Folin tube.

4. Place Folin tube in 50°C water bath to evaporate ether. Pipsyl-chloride sinks to bottom. Add distilled water to make volume to 0.6 ml..

5. Raise temperature of water bath to boiling and agitate Folin tube to emulsify pipsyl-chloride. A hand electric sander was used for agitation. Agitate for 5 minutes.

6. Permit tube to remain in water bath for 10 minutes and then cool.

7. Add 0.2 ml. N HCl and agitate.

8. Add 2ml. chloroform and agitate.

9. Transfer aqueous layer to small test tube, add 2 ml. chloroform, agitate and transfer aqueous layer to a small test tube. Repeat same process once more.

10. Transfer chloroform fractions from the 3 tubes to a separatory funnel containing a known weight, approximately 0.1 g., of non-radio-

active pipsyl-histamine derivative dissolved in chloroform. Wash tubes with 1 ml. chloroform and transfer to a separatory funnel.

11. Wash chloroform layer 3 times with N HCl; agitate for 5 minutes and permit to settle for 15 minutes before separation. Use 20 ml. of acid for each wash.

12. Determine activity of 0.5 ml. aliquot of acid wash to find effectiveness of washing process.*

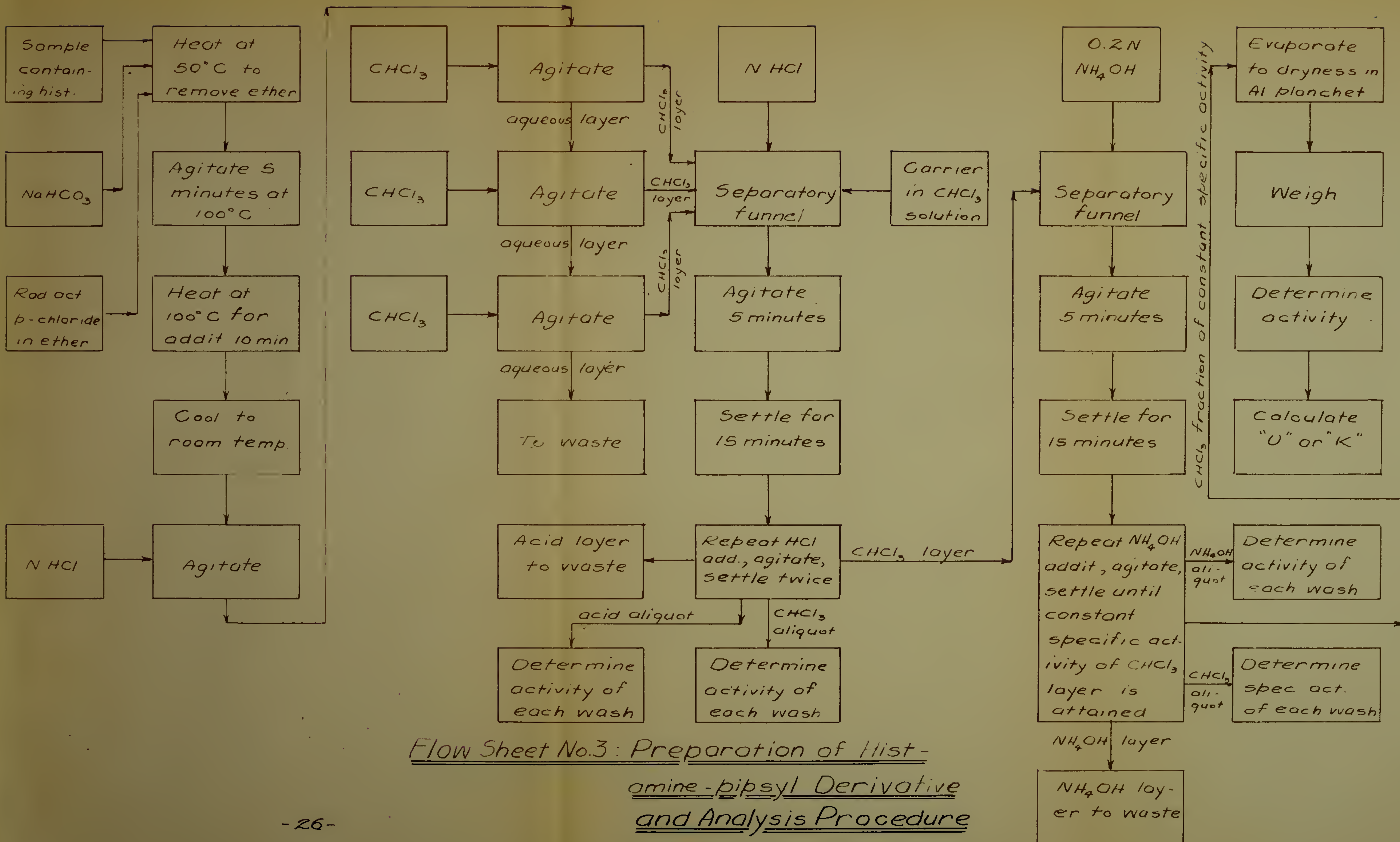
13. Wash chloroform layer with 0.2 N NH_4OH; agitate for 5 minutes and permit to settle for 15 minutes before separation. Use 20 ml. of base for each wash. Continue to wash until constant specific activity is reached.

14. Determine activity of 0.5 ml. aliquot of each base wash to find effectiveness of washing process.*

15. Determine specific activity of chloroform layer after each NH_4OH wash by evaporating 5 ml. aliquot to dryness in aluminum planchet at 55° C, followed by weighing and counting.*

16. After constant specific activity has been reached, evaporate remainder of chloroform fraction to dryness in aluminum planchet at 55°C, weigh, count and determine "U" or "K".*

*All counting was performed in flow chamber, gas mixture of 99% helium and 0.95% isobutane, using Model SC1 Autoscaler, Tracerlab.



Flow Sheet No.3: Preparation of Histamine-pipsyl Derivative and Analysis Procedure

VIII. DATA

1. Preparation of Reference Standard to Determine "U":

   a. Quantity of histamine used: 2.5μg.

   b. Quantity of carrier added: 0.104 g.

   c. Activity per 0.5 ml. aliquot of acid wash: (Background = 1.03 ct. per sec.. Counted 4096 gross counts.)

Wash	Net Counts per Second
1	910
2	55.5
3	8.0

   d. Activity per 0.5 ml. aliquot of NH_4OH wash: (Background= 1.03 ct. per sec.. Counted 4096 gross counts.)

Wash	Net Counts per Second
1	103
2	1.8
3	1.72

   e. Activity per approximately 5 ml. aliquot of chloroform layer after each NH_4OH wash: (Background = 1.03 ct. per sec., Counted 4096 gross counts.)

Aliquot Taken After NH_4OH Wash	Mg. Of Aliquot Counted	Net Counts Per Second	Net Counts Per Second Per Mg. of Aliquot	Net Counts Per Second Per μg. Histamine	'K' Counts Per Sec. Per Mole $x10^{10}$
1	8.5	35.2	4.15	172.8	1.92
2	7.5	22.3	2.98	124.0	1.38
3	7.7	22.3	2.90	120.8	1.34
*	15.4	61.1	3.96	164.5	1.83
				Ave.	1.62

*This sample represents result of addition of another 5 ml. chloroform solution to sample #3

f. Activity of remainder of chloroform fraction which was evaporated to dryness: (Background = 1.15. Counted 4096 gross counts).

Sample Number	Mg. of Sample Counted	Net Counts Per Second	Net Counts Per Second Per Mg. Of Sample	Net Counts per Second per μg. Histamine
1*	33.3	62.6	1.88	78.3
2**	33.3	97.2	2.92	121.6
3***	33.2	90.5	2.72	108.8

* Sample #1 had most of evaporated material on vertical wall of planchet; almost nothing in bottom.

** Sample #2 is sample #1 which has had the material removed from its sides and spread as uniformly as possible across bottom by dissolving in acetone and evaporating to dryness at room temperature. Sample was finally heated at 60° C for 15 minutes to complete drying operation.

*** Sample #3 is sample #2 which has been treated again with acetone and dried in an attempt to produce a more uniform distribution of evaporated material.

2. Determination of Histamine Content of Unknown Sample:

Histamine alone was included in the sample and was taken from the same solution of histamine used to prepare reference standard.

a. Quantity of histamine in sample: Unknown initially to me but later reported to be 0.75 μg..

b. Quantity of carrier added: 0.1013 g.

c. Activity per 0.5 ml. aliquot of acid wash.(Background = 1:15 ct. per sec.. Counted 4096 gross counts.)

Wash	Net Counts Per Second
1	189
2	10
3	0.8

d. Activity per 0.5 ml. aliquot of NH_4OH wash: (Background = 1.14 ct. per sec.. Counted 512 gross counts.)

Wash	Net Counts Per Second
1	----
2	0.36
3	0.48

e. Activity per approximately 5 ml. aliquot of chloroform layer after each NH_4OH wash. (Background = 1.14 ct. per sec.. Counted 4096 gross counts.)

Aliquot Taken After NH_4OH Wash	Mg. Of Aliquot Counted	Net Counts Per Second	Net Counts Per Second Per Mg. of Aliquot	Net Counts Per Second Per Unknown Sample	'U' Counts Per Second Per Mole x10^7	'w' in* μg. Histamine
1	8.0	27.3	3.42	347	3.84	2.37
2	7.4	20.4	2.76	280	3.10	1.91
3	8.3	30.6	3.69	374	4.15	2.56
3	8.4	28.5	3.40	345	3.82	2.36

* These calculated quantities are based on an average K= 1.62×10^{10}.

f. Activity of remainder of chloroform fraction which was evaporated to dryness. (Background= 1.15. Counted 4096 gross counts.)

Sample Number	Mg. of Sample Counted	Net Counts Per Second	Net Counts Per Second Per Mg. of Sample
*1	41.0	93.5	2.28
**2	40.5	89.4	2.21
***3	40.4	89.9	2.22

*Sample #1 had a concentration of evaporated material on vertical wall of planchet, however, some material was spread uniformly across bottom.

**Sample #2 is sample #1 which has had the material removed from its sides and spread as uniformly as possible across bottom by dissolving in acetone and evaporating to dryness at room temperature. Sample was finally heated at 60°C. for 15 minutes to complete drying operation.

***Sample #3 is sample #2 which had been treated again with acetone and dried in an attempt to produce a more uniform distribution of evaporated material.

IX. DISCUSSION OF DATA:

The histamine values, ranging from 1.91-2.56 μ g., obtained by the isotope derivative method for the unknown sample are between 200-400% in excess of the correct value, 0.75 μg.. Even though the error is great, the result is better than should have been expected because the actual total activity of the histamine reference standard after the final NH_4OH wash is higher than the theoretical total activity that should have been present even if the initially formed pipsyl-histamine derivative was carried through the entire operation without loss and self-absorption was absent. The basis for the above conclusion is as follows: the activity of the pipsyl-chloride added was approximately 7 counts per second per μ g. and, since 5.45 μ g. of pipsyl-chloride should combine with 1 μ g. histamine, the maximum total theoretical activity should have been approximately 95 counts per second (5.45 x 7 x 2.5); the actual total count was of the order of 300 counts per second. This indicates that some contaminant is being carried along in the chloroform fraction and is not removed by the acid and ammonium hydroxide wash. The contaminant may be pipsyl-chloride itself because it is not too easily hydrolyzed and is readily dissolved by the same solvents which dissolve the pipsyl-chloride derivative.

It is quite evident from the data that self-absorption of the soft beta-particle by the sample makes all activity readings uncertain. One is uncertain as to whether a constant molal activity is a false one due to a change in counting geometry or a true one indicating the

IX. DISCUSSION OF DATA

The histamine values, ranging from 1.11-2.90 μg., obtained by the isotope derivative method for the unknown sample are between 200-400% in excess of the correct value, 0.73 μg.. Even though the error is great, the result is better than should have been expected because the actual total activity of the histamine reference standard after the final NH_4OH wash is higher than the theoretical total activity that should have been present even if the initially formed pipsyl-histamine derivative was carried through the entire operation without loss and self-absorption was absent. The basis for the above conclusion is as follows: the activity of the pipsyl-chloride added was approximately 7 counts per second per μg. and, since 5.42 μg. of pipsyl-chloride should combine with 1 μg. histamine, the maximum total theoretical activity should have been approximately 84 counts per second (5.42 x 7 x 2.2); the actual total count was of the order of 300 counts per second. This indicates that some contaminant is being carried along in the chloroform fraction and is not removed by the acid and ammonium hydroxide wash. The contaminant may be pipsyl-chloride itself because it is not too easily hydrolyzed and is readily dissolved by the same solvents which dissolve the pipsyl-chloride derivative.

It is quite evident from the data that self-absorption of the soft beta-particle by the sample makes all activity readings uncertain. One is uncertain as to whether a constant molal activity is a false one due to a change in counting geometry or a true one indicating the

absence of contaminants; whether the observed activity is directly proportional to the quantity of material present or is less by the amount being self-absorbed. The data indicate that even when the amount of solid material in the planchet was increased from 7.7 mg. to 15.4 mg., the counting rate per mg. increased from 2.19 to 3.96, a 36% change, due to a change in the counting geometry. The data also show that when 33.3 mg. of material are re-distributed more uniformly over the counting surface, the counting rate per mg. increased from 1.88 to 2.82, a 54% increase. The magnitude of the error introduced by self-absorption has been illustrated by a study of the measured activity of S-35 in barium sulfate of constant specific activity (22):

Mg. per sq. cm. $BaSO_4$	Activity
2.5	900
5.0	1600
7.5	2200
10.0	2500
15.0	2800
20.0	3000
40.0	3100

The data above reveal that even with a surface density as low as 5 mg. per sq. cm. the activity ceases to be linear. In the experiment performed, the surface density of the evaporated pipsyl-histamine derivative, based upon the total area of the planchet, was always less than 5 mg. per sq. cm.. However, the evaporated material could not be spread uniformly over the bottom, the sample showing a tendency to concentrate in small clumps during the evaporation process.

X. CONCLUSIONS:

1. Pipsyl-chloride, tagged with radioactive S-35, can be successfully prepared from radioactive sulfanilic acid at the 0.0005 mole level. Forty-four percent of the initial activity of the sulfanilic acid was contained in the final product. There is reasonable evidence to believe that one equivalent of histamine and two equivalents of pipsyl-chloride react to form a pipsyl-histamine derivative which is insoluble in water, acid and base but is sparingly soluble in chloroform, acetone, and toluene. Labelled with a suitable radioactive isotope and employed with a suitable solvent, the pipsyl-histamine derivative holds promise for use as a tool to determine histamine quantitatively by the isotope derivative method employing the carrier technic.

2. Chloroform is not a suitable solvent for extraction of the reaction mixture because it dissolves radioactive contaminants as well as the pipsyl-histamine derivative.

3. In employing the isotope derivative method for quantitative analysis using the added carrier technic, it is highly desirable to be able to recover the component sought from the solvent by a method other than evaporation. The separation will provide a possible means of freeing the desired component from contaminants.

4. When the isotope derivative method with carrier technic is employed in quantitative determinations, S-35 is not a suitable tag, due to self-absorption of the soft beta-emission, unless the method is modified according to a technic patterned after that of Henriques et al (23)

to give reliable results. In Henriques' method the sulfur is converted to the sulfate, precipitated as the benzidine sulfate, dried, weighed and counted. In order to arrive at the true activity of the sample counted, a correction factor based upon the sample surface density is applied to the observed activity.

5. S-35 could be used to advantage as a tag in the isotope derivative method without carrier being added in which the derivative sought is recovered by paper chromatography. It was discovered that when a mixture of histamine, histidine and arginine in solution were placed on a paper chromatogram and developed with isopentanol saturated with 2N ammonium hydroxide for a period of 10 hours, the R_f factor for histamine was 0.41 and zero for histidine and arginine. Since the distance separating histamine from the basic amino acids was relatively great, it is possible that this method could be used to separate the pipsyl-derivatives of these compounds from each other even though the compounds are made more similar by the addition of the pipsyl-group.

6. Pipsyl-chloride appears to be specific for mono-amino acids, the di-amino derivatives being non-recoverable by the methods used here.

7. S-35 is an ideal radioactive isotope to work with due to its relatively long half-life, 87.3 days, and its weak beta-emission which is shielded out by the laboratory glassware.

XI. BIBLIOGRAPHY:

1. Alexander, H. L.. Synopsis of Allergy. The C. V. Mosby Co., St. Louis, 1947.

2. Behrens, C. F. (editor). Atomic Medicine. Thomas Nelson and Sons, 1949.

3. Rose, B.. Role of Histamine in Anaphylaxis and Allergy. Am. J. Med. 3, 545 - 559 (1947).

4. Hanke, M. T. and Koessler K. K.. A Method for the Quantitative Colorimetric Estimation of Histamine in Protein and Protein Containing Matter. J. Biol. Chem. 43, 543 - 556 (1920).

5. Baraud J., Genevois L., Mandielon G., and Ringenbach G.. Dosage de l' Histamine dans le Sang au Cours de Diverses Intoxications. Compt. rend. acad. Sci. 222, 760 - 761 (1946).

6. Rosenthal, S. M. and Tabor, H.. An Improved Colorimetric Method for the Estimation of Histamine. J. Pharmacol. Exp. Therap. 92, 425 - 431 (1948).

7. McIntire F. C., Roth L. W., and Shaw J. L.. Purification of Histamine for Bioassay. J. Biol. Chem. 170, 537 - 544 (1947).

8. McIntire F. C., Sproull M., and White F. B.. A New Chemical Method for the Detection of Histamine in Biological Materials. Fed. Proc. 8, 102 (1949).

9. Lubschez, R.. Chemical Determination of Histamine in Human

Blood. J. Biol. Chem. 183, 731 - 738 (1950).

10. Urbach, K. F. and Giscafre, L., Identification of Histamine in Blood by Paper Chromatography. Proc. Soc. Exp. Biol. and Med. 68, 430 - 431 (1948).

11. McIntire F. C., Roth L. W., and Shaw J. L.. The in vitro Release of Histamine from the Blood Cells of Sensitized Rabbits. Am. J. Physiol. 159, 332 - 336 (1949).

12. Code, C. F.. Histamine in Blood. Physiol. Rev. 32, 47 - 66 (1952).

13. Gaddum, J. H.. Histamine. Brit. Med. J. 1, 867 -873 (1948).

14. Keston A. S., Udenfriend S., and Cannan R. K. . A Method for Determination of Organic Acids in Form of Isotopic Derivatives. J. Amer. Chem. Soc. 71, 249 - 257 (1949).

15. Adams R., Adkins H., Blatt A. H., Cope A. C., Mc Grew F. C., Nieman C., and Snyder H. R. (editors). Organic Reactions Vol. 2. John Wiley and Sons, Inc., New York, 1944.

16. Shober, W. B.. On the Decomposition of Diazo Compounds. Am. Chem, J. 15, 379 -391 (1893).

17. Weygand, C.. Organic Preparations. Interscience Publishing Co., Inc., New York, 1945.

18. Gilman, H. and Blatt A. H.. Organic Synthesis. Vol. I. Second Edition. John Wiley and Sons, Inc., New York, 1941.

19. Lenz, W.. Ber. 10, 1135-1137 (1877).

20. Stein, W. H. and Moore, S.. The Use of Specific Precipitants in the Amino Acid Analysis of Proteins. Ann.N. Y. Acad. Sc. 47, 95 - 119 (1947).

21. Doherty D. G., Stein W. H., and Bergman M.. Aromatic Sulfonic Acids as Reagents for Amino Acids. J. Biol. Chem. 135, 487 - 496 (1940).

22. Graf W. L., Comar C. L., and Whitney I. B.. Relative Sensitivities of Windowless and End-Window Counters. Nucleonics 9 No. 4, 22 - 27 (1951).

23. Henriques F. C. Jr., Kistiakowsky G. B., Margnetti C., and Schneider W. G.. Analytical Procedure for Measurement of Long-Lived Radioactive Sulfur, S-35, with a Lauritzen Electroscope and Comparison of Electroscope with Special Geiger Counter. Ind. Eng. Chem., Anal. Ed. 18 No. 6, 349 - 353 (1946).

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